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ORIGINAL ARTICLE

Gab2 and Src co-operate in human mammary epithelial cells to promote growth factor independence and disruption of acinar morphogenesis

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The Gab2 docking protein is a target of several oncogenic protein tyrosine kinases and potentiates activation of the Ras/extracellular signal regulated kinase and phosphatidylinositol 3-kinase (PI3-kinase) pathways. Since Gab2 is phosphorylated by c-Src, and both proteins are overexpressed in breast cancers, we have determined the biological consequences of their co-expression in the immortalized human mammary epithelial cell line MCF-10A. While overexpression of c-Src did not affect acinar morphogenesis or growth factor dependence in three-dimensional culture, c-Src co-operated with Gab2 to promote epidermal growth factor (EGF)-independent acinar growth. In contrast, expression of v-Src or the activated mutant c-SrcY527F led to a spectrum of aberrant phenotypes ranging from spheroids with incomplete luminal clearance to highly disrupted, dispersed structures. Gab2 co-expression shifted the phenotypic distribution towards the dispersed phenotype, an effect not observed with a Gab2 mutant unable to bind the p85 subunit of PI3-kinase (Gab2Δp85). In v-Src-expressing cells, Gab2, but not Gab2Δp85, significantly decreased E-cadherin adhesive strength without altering its surface expression. Gab2 associated with E-cadherin in the presence and absence of v-Src, indicating that the ability of Gab2 to weaken the strength of cell–cell contacts may reflect enhanced activation of PI3-kinase at adherens junctions. Gab2 also increased migration and invasion of these cells in transwell assays, but these effects were p85-independent. Overall, these findings demonstrate a novel mechanism whereby Gab2 may promote metastatic spread and indicate that Gab2 may play several roles during breast cancer progression.
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Introduction

The docking protein Grb2-associated binder (Gab)2 is recruited and tyrosine phosphorylated following activation of diverse receptor types, including specific receptor tyrosine kinases (RTKs), B- and T-cell antigen receptors, RANK and β_1-integrin (Nishida *et al*., 1999; Yu *et al*., 2002; Wada *et al*., 2005). Once phosphorylated, Gab2 binds several effectors, including the protein tyrosine phosphatase Shp2 and the p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase). Association with the latter two proteins potentiates Ras/extracellular signal regulated kinase (Erk) and PI3-kinase/Akt signalling (Gu and Neel, 2003). Although RTKs may directly phosphorylate Gab2, receptor-associated tyrosine kinases also play an important role. For example, in haematopoietic cells, Syk, ZAP-70 and Src family kinases may mediate this modification, depending on the stimulus (Yamasaki *et al*., 2001; Podar *et al*., 2004; Yu *et al*., 2006). In primary hepatocytes, Gab2 is tyrosine phosphorylated by c-Src in response to epidermal growth factor (EGF) treatment, and this leads to PI3-kinase activation and DNA synthesis (Kong *et al*., 2003).

Importantly, Gab2 is implicated in oncogenic signalling and human cancer. Gab2 transforms fibroblasts when relieved from Akt-mediated negative feedback (Lynch and Daly, 2002) and is essential for the transforming activity of oncogenes such as v-Sea (Ischenko *et al*., 2003), Bcr-Abl (Sattler *et al*., 2002) and Shp2 E76K, a Shp2 hyperactive mutant (Mohi *et al*., 2005). It is also overexpressed in breast cancer cell lines and primary breast cancers (Daly *et al*., 2002; Bentires-Alj *et al*., 2006; Brummer *et al*., 2006) and supporting a functional role for Gab2 in the development and/or progression of this disease, Gab2 overexpression increases proliferation and promotes EGF-independence of MCF-10A immortalized mammary epithelial cells in three-dimensional culture (Bentires-Alj *et al*., 2006; Brummer *et al*., 2006). In addition, Neu-induced mammary tumourigenesis in mice is attenuated in a Gab2-null background and potentiated by Gab2 overexpression (Bentires-Alj *et al*., 2006), and Gab2 ablation significantly impairs lung metastasis of mammary tumours induced by an oncogenic form of Neu (Ke *et al*., 2007). The functional contribution of Gab2 to signalling from other kinases commonly deregulated in breast cancer is currently unknown.

Increased expression and/or activity of the protein tyrosine kinase c-Src commonly occurs in several human cancers such as those of the breast, colon and pancreas (Irby and Yeatman, 2000). Inhibition of c-Src function or expression in the MCF-7 human breast cancer cell line has identified roles for this kinase in promoting cell proliferation and migration, as well as tumourigenesis in nude mice (Gonzalez *et al*., 2006). Increased Src activity is associated with reduced cell–cell adhesion and invasiveness. Expression of activated Src mutants in epithelial cells leads to the endocytosis and down-regulation of E-cadherin, and hence disassembly of adherens junctions (Frame, 2004). This suggests that increased Src activity may contribute to the metastatic spread of epithelial cancers. Interestingly, expression of phosphatase and tensin homologue deleted from chromosome ten (PTEN), in v-Src-expressing epithelial cells stabilizes E-cadherin junctions and attenuates cell scattering and invasiveness, indicating that enhanced PI3-kinase signalling may underlie these biological effects of v-Src (Kotelevets *et al*., 2001).

Since Gab2 and c-Src are functionally linked (Kong *et al*., 2003), and both implicated in breast cancer (Irby and Yeatman, 2000; Daly *et al*., 2002; Bentires-Alj *et al*., 2006; Brummer *et al*., 2006; Ke *et al*., 2007), we have determined the biological consequences of their combined overexpression using the human mammary epithelial cell line MCF-10A. When cultured on Matrigel, these cells form acini that model mammary lobules *in vivo*, comprising of a single cell layer with apicobasal polarity surrounding a hollow lumen. This provides a powerful model system for characterizing the biological activity of oncogenes, since depending on the proliferative, anti-apoptotic and/or invasive signals provided, parameters such as acinar size, luminal clearance or integrity may be affected (Debnath *et al*., 2003a). Our findings indicate that while Gab2 co-operates with overexpressed c-Src to enhance proliferative signalling, co-expression of Gab2 with activated Src mutants promotes acinar disruption and this reflects a novel role of Gab2 in modulating E-cadherin function via PI3-kinase.

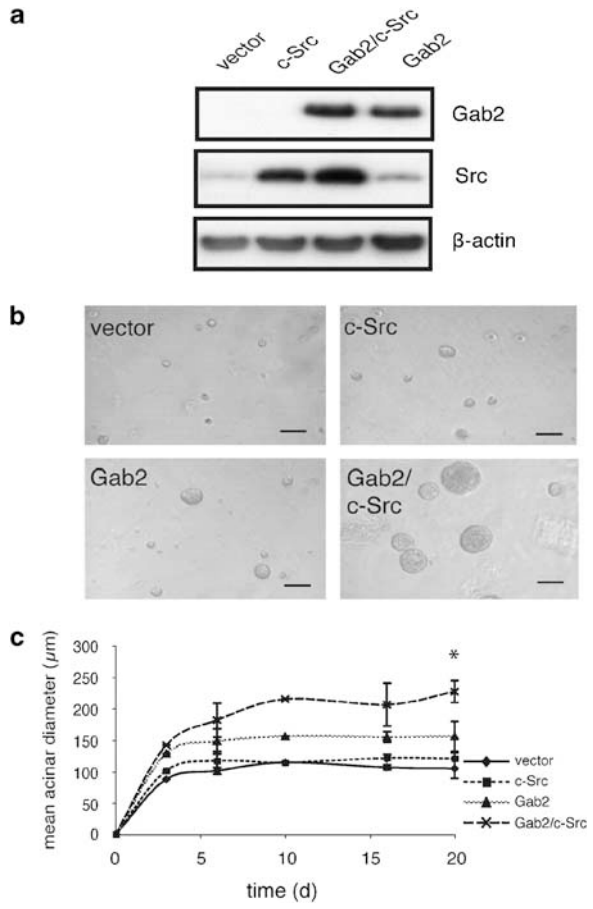

Figure 1 Effects of Gab2 and c-Src co-expression in MCF-10A cells in three-dimensional Matrigel culture. (**a**) Expression levels of Gab2 and c-Src in monolayer culture. Cell lysates were western blotted as indicated, with β-actin as a loading control. (**b**) Representative phase-contrast images of vector control, c-Src-, Gab2- and Gab2/c-Src-overexpressing acini at day 20, maintained in medium supplemented with insulin. Scale bar, 250 μm. (**c**) Effect of Gab2/c-Src co-expression on acinar diameter. Acini were maintained as described in (b). The mean acinar diameter was determined at days 6, 16 and 20 in three independent experiments, with 100 structures analysed per time point. Error bars indicate s.e. $*P<0.05$; significant difference between Gab2- and Gab2/c-Src-infected cells.

Results

Effect of combined overexpression of Gab2 and c-Src on MCF-10A acinar morphogenesis

To determine the consequences of upregulation of Gab2 and c-Src, MCF-10A cells were infected with retroviruses encoding these proteins alone or in combination (Figure 1a). c-Src overexpression had no effect on acinar growth or morphogenesis, regardless of the growth factor conditions employed. Like vector controls, these cells only formed small, disorganized clusters under insulin-only conditions. In contrast, MCF-10As overexpressing both c-Src and Gab2 grown in insulin-only cultures exhibited a marked growth promotion compared to Gab2-overexpressing acini, this being evident as early as day 3, and significant at day 20 (Figures 1b and c). These acini exhibited normal lumen formation, since increased proliferation within the structures (as indicated by Ki67 staining) was opposed by enhanced rates of apoptosis (as evidenced by staining for active caspase 3) (data not shown). Therefore, c-Src and Gab2 co-operate in MCF-10A cells to promote EGF-independent acinar growth.

Western blot analysis of acini maintained under insulin-only conditions revealed that co-expression of c-Src with Gab2 led to a significant increase in Gab2 tyrosine phosphorylation (Figure 2a). Expression of c-Src caused a slight increase in Erk activation (Figure 2b) and a significant enhancement of Stat5 phosphorylation (Figure 2c). Gab2 markedly enhanced Erk activation, but no effect was observed on Stat5. Co-expression of c-Src and Gab2 resulted in a modest

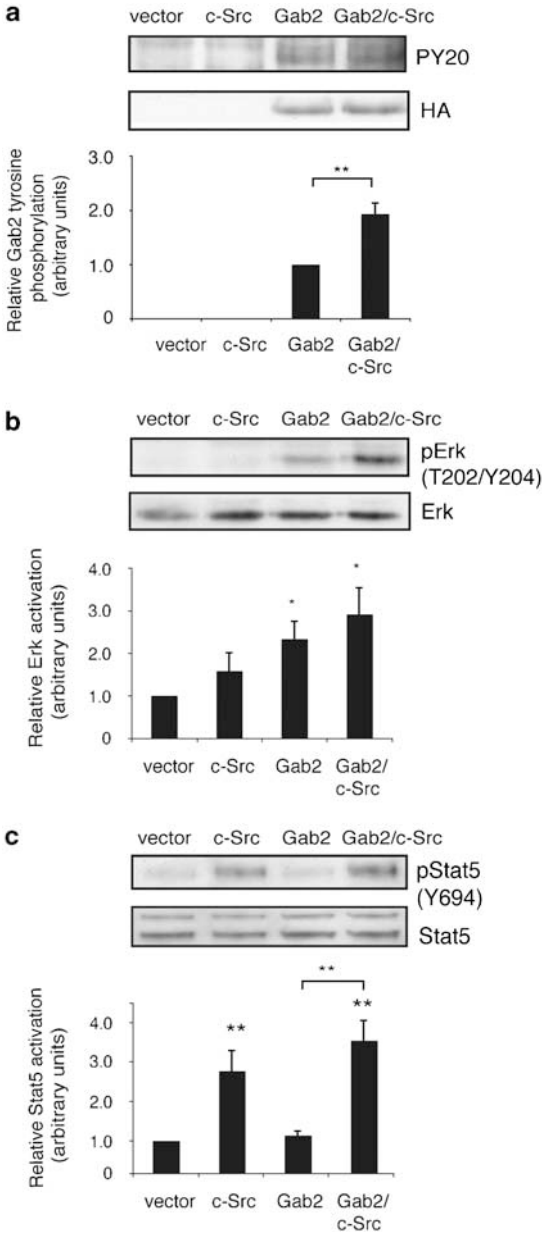

Figure 2 Signalling in Gab2/c-Src co-expressing acini. Acini were grown in full medium until day 3 and then changed to medium supplemented with insulin. Acini were isolated on day 4 and lysates were subjected to western blotting. (**a**) Effect of Gab2/c-Src co-expression on Gab2 tyrosine phosphorylation. The PY20 blot shows a band of approximately 95 kDa representing tyrosine-phosphorylated Gab2, as confirmed by reprobing with an anti-HA antibody. Effect of Gab2/c-Src co-expression on activation of Erk (**b**) and Stat5 (**c**). The graphs show Gab2 tyrosine phosphorylation (a) or Erk (b) and Stat5 (c) activation normalized for total protein levels, with the level for cells expressing Gab2 only (a) or vector controls (b, c) arbitrarily set at 1.0. The data are derived from three independent experiments. The error bars indicate s.e. $*P<0.05$, $**P<0.01$, representing significant differences versus vector or otherwise as indicated.

increase in activation of Erk compared to Gab2 overexpression alone, and in Stat5 activation compared to that of c-Src, but these differences were not significant. Although Gab2 overexpression increases Akt activation in the presence of EGF (Brummer *et al*., 2006), we were unable to detect effects of c-Src, Gab2 or the two in combination on Akt activation under insulin-only conditions (data not shown).

v-Src or c-SrcY527F expression disrupts acinar morphogenesis

Since increased Src activity has been detected in human breast cancers (Irby and Yeatman, 2000), we next utilized two Src mutants—the viral oncoprotein v-Src and the activated mutant c-SrcY527F (Reynolds *et al*., 1987; Frame, 2004). Expression levels of retrovirally infected MCF-10A cell lines are shown in Figure 3a. Three-dimensional culture of these cells generated aberrant phenotypes that fell into three main categories (Figure 3b). Spheroidal structures appeared normal under phase contrast microscopy, possessing a smooth, even surface (Figure 3b, left panel). However, the central matrix-detached cells of these spheroids exhibited reduced rates of apoptosis and thus did not clear the luminal space (Figure 3c, lower right panel). Discohesive structures were comprised of cells that were clustered together but lacked a unified smooth appearance, had aberrant laminin V deposition and did not undergo luminal clearance (Figure 3b, middle panel; Figure 3c, lower left panel). In dispersed structures, cells detached from the loosely clustered main body of the structure and were distributed throughout the surrounding Matrigel (Figure 3b, right panel; Figure 3c, lower right panel). Staining of spheroidal-type structures indicated that E-cadherin was predominantly localized to cell–cell junctions. However, in discohesive structures, staining was weaker and largely diffuse and cytoplasmic, although some plasma membrane staining was still evident. This staining pattern did not alter further in dispersed structures. All three phenotypic categories were observed in cultures of cells expressing v-Src or c-SrcY527F, regardless of the growth factor conditions employed (data not shown).

Gab2 co-operates with v-Src or c-SrcY527F to promote acinar growth and disruption

Spheroidal structures expressing v-Src or c-SrcY527F were larger than the corresponding vector control acini, and the mean spheroidal diameter was significantly augmented when Gab2 was co-expressed (Figure 4a). This effect was observed under all growth factor conditions (data not shown). Next, we determined whether Gab2 altered the phenotypic distribution of v-Src- or c-SrcY527F-expressing structures. The proportion of dispersed structures was significantly increased when either mutant was co-expressed with Gab2. This effect was associated with a corresponding decrease in the proportion of spheroidal structures, indicating that Gab2 co-expression shifted the entire population towards the more advanced phenotype (Figures 4b and c), and was observed in the presence or absence of EGF (data not shown). These results demonstrate that Gab2 can potentiate both the proliferative effects and disruption

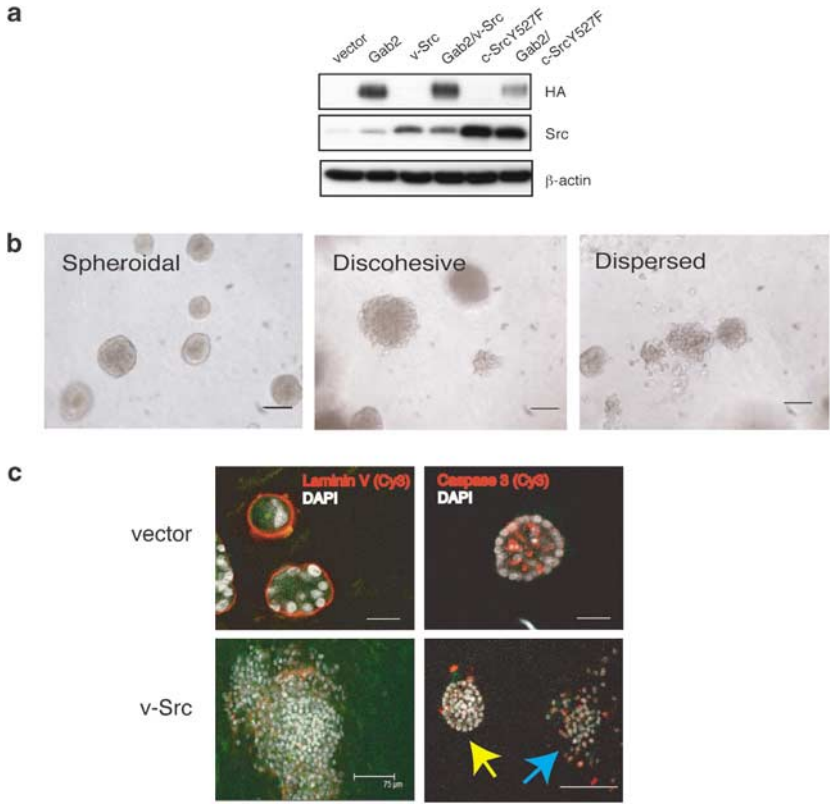

Figure 3 Expression of activated Src mutants in MCF-10A cells results in a disruption of acinar morphogenesis. (**a**) Expression levels of activated Src mutants and Gab2. (**b**) Representative phase-contrast images of v-Src-expressing cells cultured on Matrigel. Spheroidal, discohesive and dispersed phenotypes are depicted. Scale bar, 500 μm. (**c**) Representative confocal images displaying morphology of vector control or v-Src-expressing structures. Structures were stained with antibodies against laminin V (left panels) or active Caspase-3 (right panels). Yellow and blue arrows in the bottom right panel indicate spheroidal and dispersed structures, respectively. Scale bars, 75 μm. For (b and c), cultures were maintained in medium supplemented with EGF and insulin and images obtained at day 10.

of structural integrity that results from v-Src or c-SrcY527F expression.

The impact of Gab2/v-Src co-expression on cellular signalling was determined for cultures supplemented with both EGF and insulin. As described previously (Brummer *et al*., 2006), Gab2 significantly increased Akt S473 phosphorylation under these conditions. Expression of v-Src caused a robust increase in Akt activation, and this was enhanced by Gab2 co-expression (Figure 5a). Surprisingly, v-Src alone decreased Erk activation and also slightly attenuated the increased Erk phosphorylation observed in the presence of Gab2 (Figure 5b). Similar to results obtained for c-Src (Figure 2c), v-Src markedly enhanced Stat5 phosphorylation and this was increased further by Gab2 co-expression (Figure 5c). Therefore, Gab2 overexpression amplifies several signalling pathways in v-Src-expressing MCF-10A cells.

Binding of p85 is required for Gab2 to promote the v-Src-induced dispersed phenotype

In order to determine the contributions of particular Gab2/effector interactions to phenotypic outcome in Gab2/v-Src structures, we expressed v-Src with Gab2 mutants that lack binding sites for either p85 (Gab2Δp85) or Shp2 (Gab2ΔShp2). Expression levels of these cell lines are shown in Figure 6a. Immunoprecipitations from EGF-stimulated monolayer cultures were performed to confirm the binding activity of these mutants (Figure 6b). As expected, Gab2Δp85 was defective for p85 binding but retained Shp2 association. However, Gab2ΔShp2 retained significant association with Shp2 when co-expressed with v-Src. While this likely reflects an indirect mechanism, we decided to discontinue use of this mutant due to its ambiguous behaviour.

Spheroids co-expressing v-Src and Gab2Δp85, while larger than those expressing v-Src alone, did not attain the diameter of those with v-Src and wild-type Gab2 (Figure 6c), indicating that p85 binding is not the only Gab2 effector pathway that contributes to acinar growth promotion. This is consistent with our previous data for acini expressing Gab2 alone, which indicated roles for both p85 binding and Erk activation (Brummer *et al*., 2006). We then determined the impact of Gab2Δp85 upon phenotype distribution. Strikingly, Gab2Δp85/v-Src-expressing cultures did not exhibit the shift of the population towards the dispersed category that occurs with wild-type Gab2 co-expression

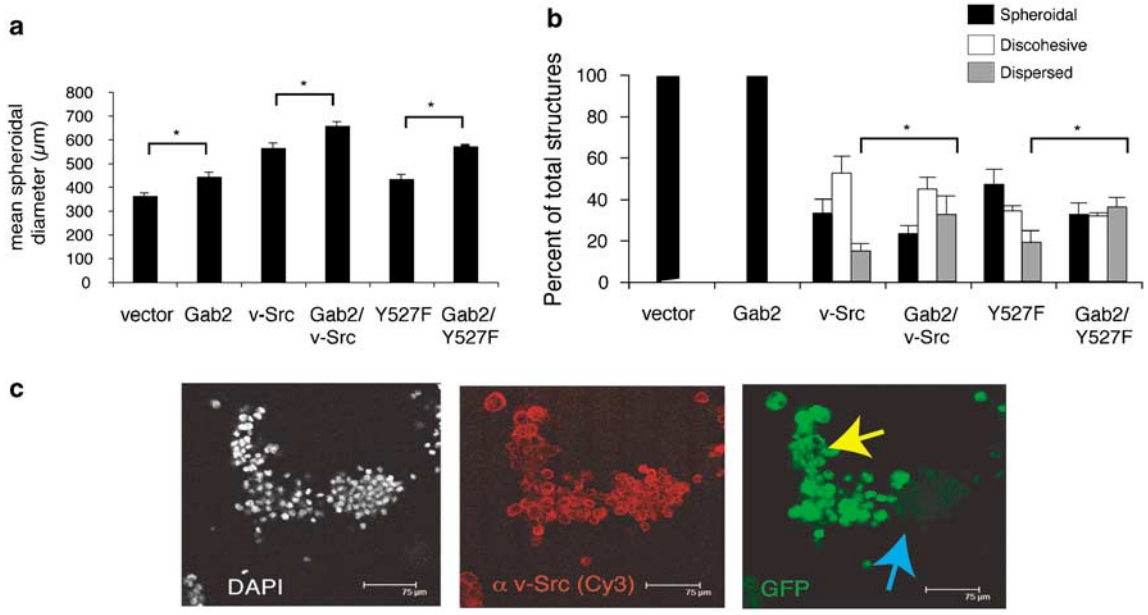

Figure 4 Co-expression of Gab2 with active Src mutants increases the proportion of dispersed structures. Effect of Gab2 on spheroidal diameter (**a**) and v-Src-induced phenotypic distribution (**b**). Structures were visualized by phase-contrast microscopy and manually scored at day 10. Data were derived from three independent experiments. For each experiment, 30 structures were analysed in (a) and 100 in (b). Error bars indicate s.e. $*P<0.05$, indicating significant difference between v-Src- and Gab2/v-Src- or c-SrcY527F- and Gab2/c-SrcY527F-infected cells. (**c**) Confocal images of Gab2/v-Src co-expressing structures at day 10 with comparable v-Src expression (middle panel) but contrasting Gab2 expression (right panel). The yellow arrow indicates a dispersed structure with high Gab2 expression; the blue arrow indicates a discohesive structure with low Gab2 expression. The structures were stained with an antibody against Src, the nuclei were visualized using DAPI stain (left panel) and GFP fluorescence serves as a surrogate marker for Gab2 expression. For (a–c), structures were maintained as described in Figure 3. Scale bar, 75 μm.

(Figure 6d). Rather, these cultures displayed the same phenotypic distribution as that observed for v-Src expression alone. Therefore, p85 binding is essential for Gab2 to promote the v-Src-induced dispersed phenotype in this system.

Gab2 promotes v-Src-induced inhibition of E-cadherin adhesion

As Gab2 co-expression with v-Src decreased the proportion of spheroidal structures, we tested whether this resulted from changes to E-cadherin-based adhesive strength by plating cells on hE/Fc-coated substrata and measuring their resistance to detachment (Verma *et al*., 2004; Shewan *et al*., 2005). While Gab2 alone did not alter this parameter, it was lowered by expression of v-Src (Figure 7a). However, Gab2 co-operated with v-Src to further reduce adhesive strength, and importantly, this effect was not observed with the Gab2Δp85 mutant. Therefore, the relative abilities of Gab2 and Gab2Δp85 to promote acinar disruption (Figure 6d) are tightly correlated with their effects on E-cadherin-based adhesion.

To investigate the mechanistic basis for this effect, we first determined the expression of E-cadherin and two other components of the adherens junction complex, β- and α-catenin, in RIPA lysates. Expression of both E-cadherin and α-catenin was significantly reduced in the presence of v-Src, but Gab2 or Gab2Δp85 did not modulate expression of these proteins further. Levels of β-catenin were similar in all cell pools (data not shown). Next, we assayed surface E-cadherin expression. Confluent cells were trypsinized in the presence of calcium, thus protecting the E-cadherin extracellular domain, or in the absence of calcium, rendering this region susceptible to tryptic cleavage. Western blotting was then used to assay total cellular levels of E-cadherin (Trypsin + Ca) and the intracellular pool (Trypsin + EDTA), enabling indirect assessment of surface levels. This revealed that while v-Src expression resulted in a reduction in surface E-cadherin, co-expression of Gab2 or Gab2Δp85 did not lower this further (Figure 7b).

E-cadherin functions in close co-operation with the actin cytoskeleton (Mege *et al*., 2006). Since co-immunoprecipitation of E-cadherin with β- and α-catenin was unaffected by v-Src and/or Gab2 expression (data not shown), we indirectly assessed possible changes in cytoskeletal association of the cadherin–catenin complex by determining the proportion of E-cadherin in the triton-insoluble fraction of cells (Shore and Nelson, 1991; Wrobel *et al*., 2004). The amount of E-cadherin in this fraction was markedly reduced upon expression of v-Src, and co-expression of Gab2 lowered this further (Figure 7c). While the former effect may simply reflect a decrease in total E-cadherin levels, the latter occurs without a further change in E-cadherin expression. However, since Gab2Δp85 also reduced the amount of E-cadherin in the triton-insoluble fraction, this effect of Gab2 cannot explain the changes in E-cadherin-based adhesion observed (Figure 7a).

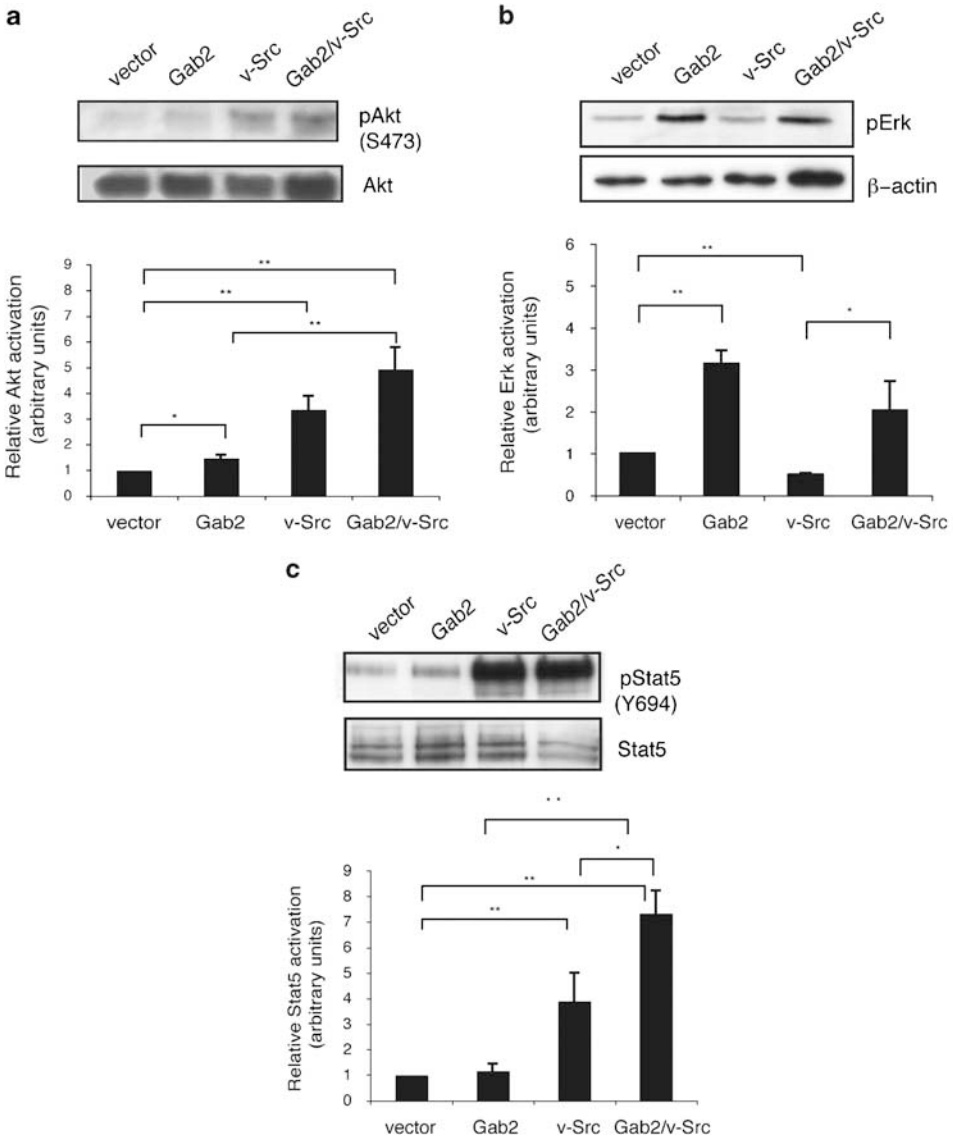

Figure 5 Signalling in Gab2/v-Src co-expressing structures. Effect of Gab2/v-Src co-expression on activation of Akt (**a**), Erk (**b**) and Stat5 (**c**). Structures were maintained as described in Figure 3, isolated at day 4 and lysates subjected to western blotting. Graphs show relative activation normalized to total protein levels, with the level for vector cells arbitrarily set at 1.0. Data are derived from three independent experiments. Error bars indicate s.e. $*P<0.05$ and $**P<0.01$, designating significant differences as indicated.

Finally, we determined whether Gab2 co-localizes or interacts with components of adherens junctions. First, we determined the subcellular localization of Gab2 in MCF-10A acini by confocal microscopy. This revealed that in acini overexpressing Gab2 alone (data not shown) or with c-Src (Figure 8a), Gab2 predominantly localized to cell–cell junctions. Association of Gab2 and/or v-Src with E-cadherin was then assayed. When expressed alone, Gab2 and v-Src co-immunoprecipitated with E-cadherin (Figure 8b). Furthermore, although the amount of Gab2 co-immunoprecipitated with E-cadherin was less, complex formation between these three proteins could be detected in Gab2/v-Src co-expressing cells. These data suggest that modulation of E-cadherin-based adhesive strength by Gab2 may stem from localized signalling by this docking protein at adherens junctions.

Recruitment of p85 is not required for Gab2 to enhance the migration and invasion of v-Src-expressing cells

Metastatic spread of epithelial cancers involves multiple steps, including dissolution of cell–cell contacts, migration of cells and their invasion through basement membrane. Therefore, we performed transwell assays to determine whether Gab2-co-expression altered the migratory and/or invasive potential of v-Src-expressing cells. Vector control and Gab2-overexpressing cells exhibited very low migration rates and did not invade (Figures 8c and d). Migration and invasion were significantly enhanced by v-Src expression and increased further upon Gab2 co-expression. However, the latter effect was also observed with Gab2Δp85. This indicates that while PI3-kinase signalling downstream of Gab2 attenuates E-cadherin-based adhesion of v-Src-expressing cells, it is not required for Gab2 to amplify their migratory and invasive potential.

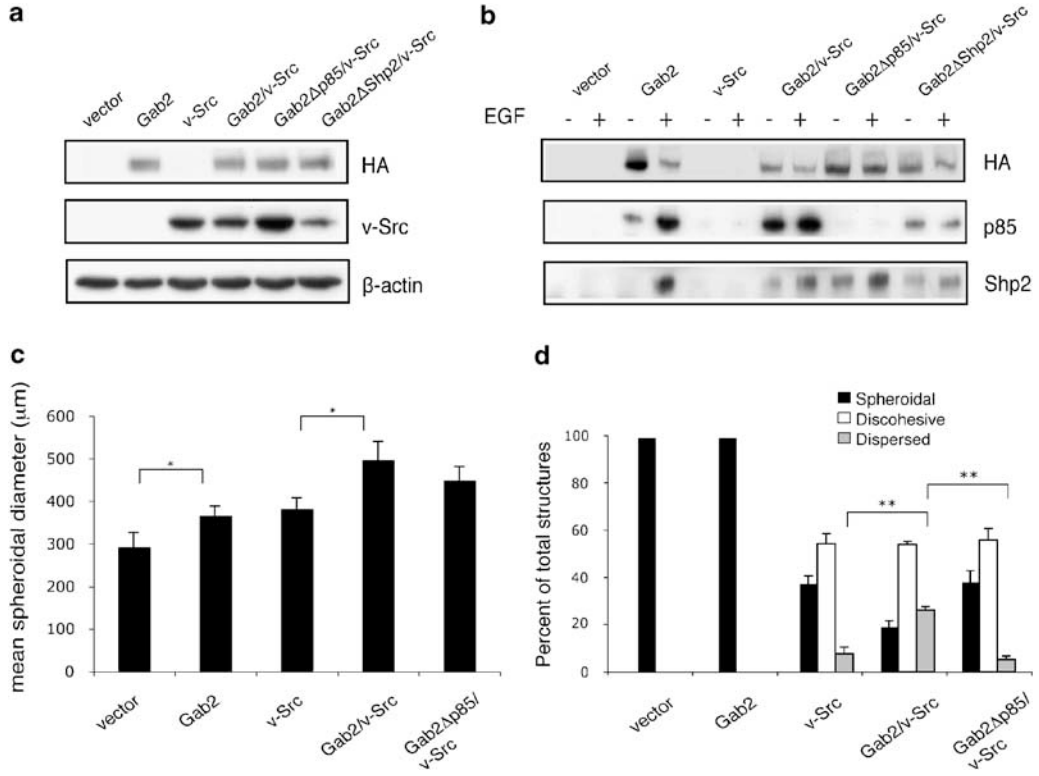

Figure 6 Binding of p85 is required for Gab2 to promote the v-Src-induced dispersed phenotype. (**a**) Expression levels of v-Src and Gab2, Gab2Δp85 or Gab2ΔShp2 in MCF-10A cells in monolayer culture. (**b**) Binding activity of the Gab2Δp85 and Gab2ΔShp2 mutants in v-Src-expressing cells. Monolayer cultures expressing the indicated vectors were stimulated with EGF and anti-HA immunoprecipitates western blotted with antibodies against p85, Shp2 and HA. (**c**) Effect of Gab2Δp85 on spheroidal diameter. (**d**) Effect of Gab2Δp85 on phenotype distribution. Structures in (c and d) were scored as described in Figure 4. Error bars indicate s.e. $*P<0.05$ and $**P<0.01$; representing a significant difference between indicated groups.

Discussion

Since Gab2 is overexpressed in a subset of breast cancers (Daly *et al*., 2002; Bentires-Alj *et al*., 2006), we have exploited the MCF-10A three-dimensional culture system in order to dissect the functional roles that Gab2 may play in this disease. Previously, we determined that Gab2 overexpression increases the size of acinar structures, overcomes proliferative suppression in late-stage cultures and promotes EGF-independence of the morphogenetic program (Brummer *et al*., 2006). In this study, we report that the latter effect is markedly enhanced upon co-expression with the proto-oncogene c-Src, which is also upregulated in breast cancer. Moreover, Gab2 co-operates with active forms of this tyrosine kinase to enhance dissociation of three-dimensional spheroids and the migratory and invasive potential of MCF-10A cells. These findings indicate that Gab2 may co-operate not only with receptor tyrosine kinases implicated in this disease, such as erbB2 (Bentires-Alj *et al*., 2006; Ke *et al*., 2007), but also with receptor-associated kinases of the Src family. Furthermore, they indicate that Gab2 may act both early and late in tumour progression.

Co-expression of c-Src or activated Src mutants with Gab2 led to enhanced Gab2 tyrosine phosphorylation in serum-starved cells in monolayer culture (data not shown). However, in contrast to the findings of others (Kong *et al*., 2003) we were unable to detect either form of Src in Gab2 immunoprecipitates, and *vice versa*. This suggests that in MCF-10A cells, their interaction is transient and/or indirect. Such interaction may be promoted at cell–cell junctions, based on our localization of Gab2 to these sites, and co-immunoprecipitation of both v-Src and Gab2 proteins with E-cadherin.

The ability of Gab2 to promote EGF-independent morphogenesis of MCF-10A cells likely reflects its ability to amplify multiple signalling pathways, as it is dependent on recruitment of both Shp2 and the p85 subunit of PI3-kinase (Brummer *et al*., 2006). This is also consistent with the observation that activated Akt cannot drive this process unless combined with HPV E7 or overexpressed cyclin D1 (Debnath *et al*., 2003b). Since co-expression of c-Src with Gab2 significantly enhanced Gab2 tyrosine phosphorylation, amplification of signalling downstream of Gab2 provides one explanation for the combined effect of c-Src and Gab2 on EGF independence. However, overexpression of c-Src, but not Gab2, led to a significant increase in Stat5 phosphorylation. Since Stat5 can positively regulate cyclin D1 expression and hence cell cycle progression (Clevenger, 2004), this pathway may also contribute to Gab2/c-Src co-operation. Interestingly, co-expression of Gab2 with either c-Src or v-Src further enhanced Stat5 activation.

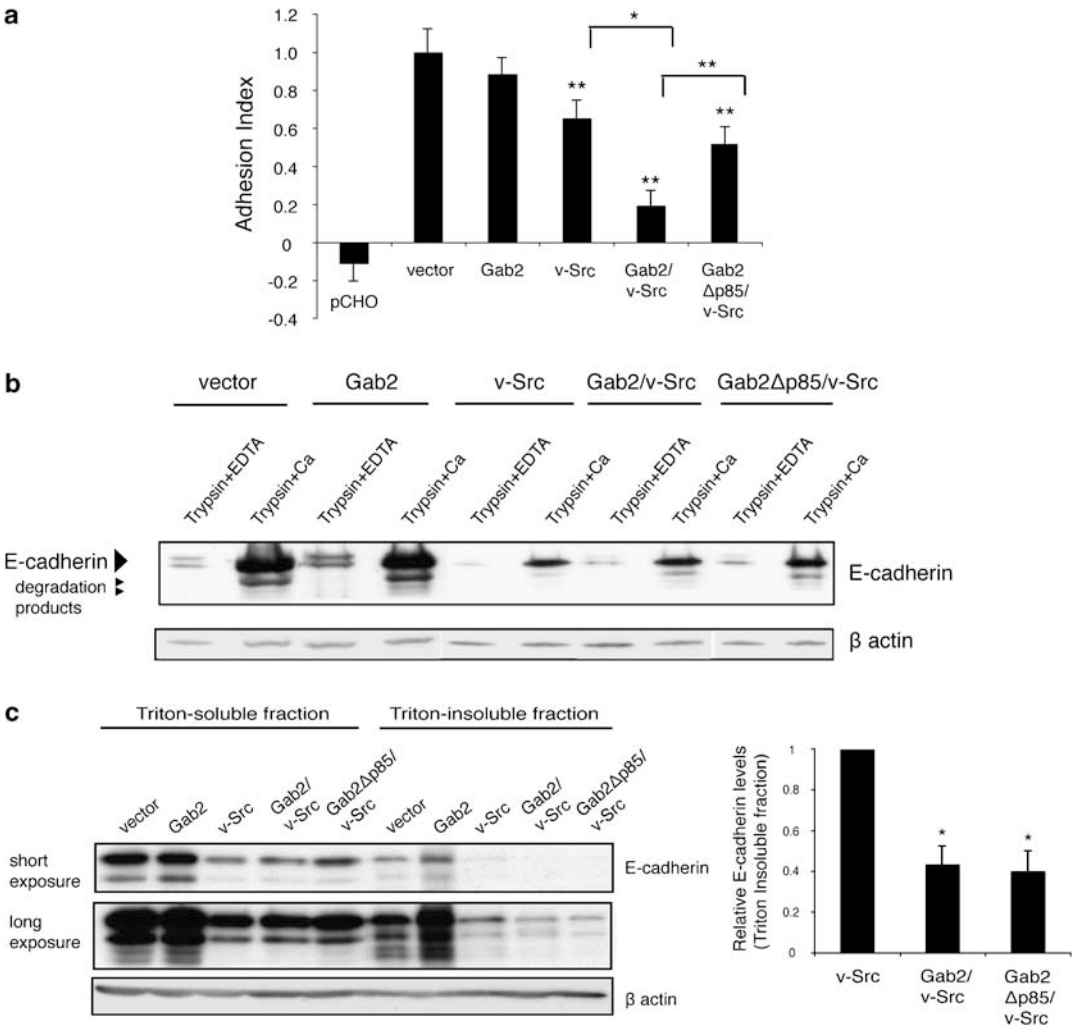

Figure 7 Effect of Gab2/v-Src co-expression on function and expression of the E-cadherin-based adherens junction complex. (**a**) Gab2 potentiates the v-Src mediated reduction in E-cadherin adhesive strength in a manner dependent upon Gab2/p85 association. Binding of cells to hE/Fc-coated planar substrata was assayed as described in Materials and methods. All data points were normalized to the average adhesion index value obtained for the vector control cell line. Error bars indicate s.e. $*P<0.01$, $**P<0.001$, representing a significant difference between vector-infected cells and the indicated group, or as indicated. (**b**) Determination of surface E-cadherin expression. Confluent cells were trypsinized in the presence or absence of calcium and levels of E-cadherin were assessed by western blotting. (**c**) Association of the E-cadherin complex with the triton-insoluble fraction of cells. The graph depicts relative E-cadherin levels within the Triton-insoluble fraction normalized to β-actin levels, with the level for v-Src cells arbitrarily set at 1.0. For (b and c), data are representative of three independent experiments. Error bars indicate s.e. $*P<0.05$, representing a significant difference between v-Src-infected cells and the indicated group.

This may result from recruitment of Stat5 to a Gab2 scaffold (Nyga *et al*., 2005), facilitating subsequent tyrosine phosphorylation of Stat5 by Src (Clevenger, 2004).

Expression of activated Src was accompanied by a robust activation of Akt, that likely reflects the ability of Src to stimulate PI3-kinase both directly (Lu *et al*., 2003) and indirectly by phosphorylation of p85 binding proteins such as c-Cbl (Kassenbrock *et al*., 2002). However, a surprising observation was that Erk activation was not increased by v-Src, but was suppressed. This effect may be mediated by crosstalk between Akt and the Ras/Erk pathway. For example, Akt can negatively regulate Raf-1 and B-Raf by direct phosphorylation (Guan *et al*., 2000). Furthermore, Akt1 negatively regulates Erk activation in IGF-1R-stimulated MCF-10A cells, and downregulation of Akt1 promotes an epithelial-mesenchymal transition (EMT) that is dependent on Akt2 expression (Irie *et al*., 2005). Consequently, amplified PI3-kinase/Akt signalling in the v-Src-expressing cells may both suppress Erk activation and promote the dispersed phenotype. This is consistent with the ability of activated Akt to promote EMT of squamous cell carcinoma cells (Grille *et al*., 2003) and of PTEN to inhibit v-Src-induced adherens junction disassembly in Madin–Darby canine kidney (MDCK) cells (Kotelevets *et al*., 2001).

The altered expression and localization of E-cadherin in discohesive structures explains the loosening of cell–cell contacts, and is consistent with the ability of v-Src to

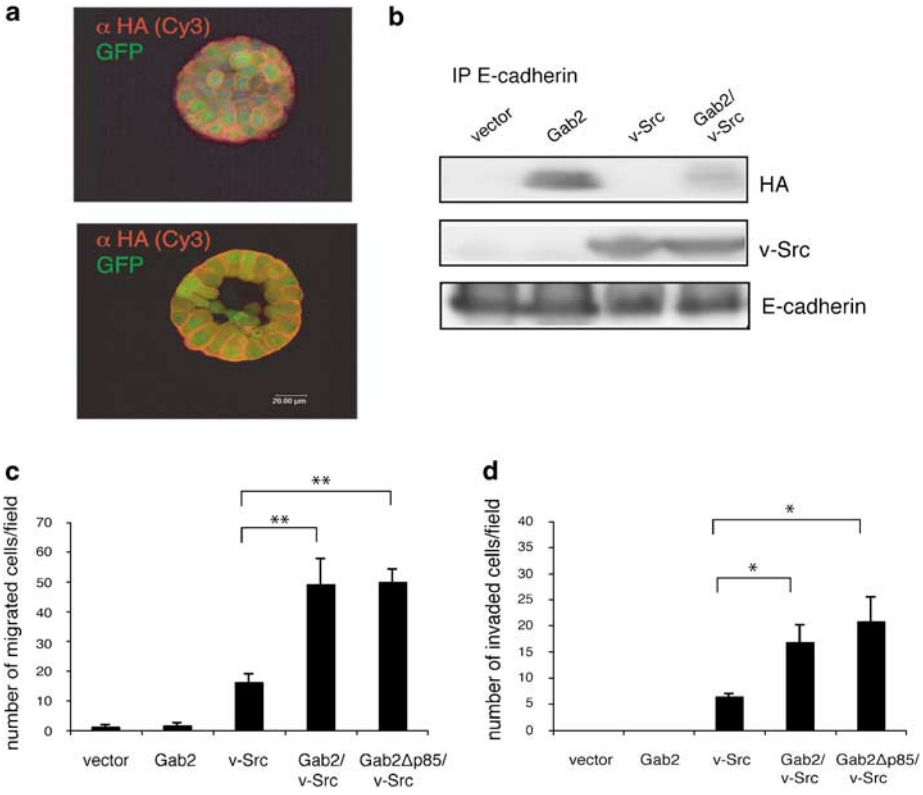

Figure 8 Gab2 associates with E-cadherin and enhances v-Src-induced migration and invasion. (**a**) Gab2 localizes to cell–cell junctions in MCF-10A acini. Images represent polar (upper panel) and equatorial (lower panel) confocal cross-sections of acini co-expressing Gab2 and c-Src at day 10 grown in medium supplemented with EGF and insulin. Acini were stained with an antibody against HA. Scale bar, 20 μM. (**b**) Gab2 and v-Src associate with E-cadherin. E-cadherin immunoprecipitates from Triton lysates were western blotted as indicated. (**c**) Effect of Gab2 on v-Src-induced cell migration. (**d**) Effect of Gab2 on v-Src-induced cell invasion. In (c and d), transwell migration and invasion assays were performed as described in Materials and methods and data are derived from three independent experiments. Error bars indicate s.e. $*P<0.05$ and $**P<0.01$, representing a significant difference between indicated groups.

promote endocytosis and degradation of E-cadherin (Frame, 2004). The dispersed phenotype may reflect a further lowering of E-cadherin-based adhesive strength via mechanisms not resolved/detected by staining, combined with the more motile and invasive phenotype conferred by v-Src expression. However, the shift in phenotypic distribution conferred by Gab2 co-expression correlates with its effect on E-cadherin-based adhesion rather than invasion, as only the former is dependent on p85-binding. The mechanism whereby Gab2 reduces E-cadherin-based adhesive strength is unclear, although modulation of cadherin function independently of changes in surface expression is not without precedent. For example, this is characteristic of the reduction in C-cadherin-mediated adhesion that occurs upon activin treatment of *Xenopus* blastomeres (Brieher and Gumbiner, 1994). One possibility is that it reflects altered lateral clustering of cadherin molecules within the plasma membrane. This is supported by the observation that while inhibition of Myosin II activity reduces E-cadherin-based cell adhesion, it does not suppress E-cadherin surface expression but instead alters the regional distribution of this molecule, abrogating the formation of E-cadherin 'macroclusters' (Shewan *et al*., 2005).

Binding of p85 was absolutely required for Gab2 to reduce the strength of E-cadherin-based adhesions between v-Src-expressing cells. This is intriguing in light of reports that depending on context, PI3-kinase signalling can play positive or negative roles in the formation of E-cadherin-based adherens junctions. Thus, while PI3-kinase signalling enhances the formation of nascent adhesive contacts following E-cadherin homophilic ligation (Kovacs *et al*., 2002; Pang *et al*., 2005), in v-Src-expressing MDCK cells it promotes junctional instability, since cell–cell aggregation can be induced in an E-cadherin-dependent manner by expressing PTEN (Kotelevets *et al*., 2001). These contrasting effects are probably explained by marked differences in signal strength and duration. Indeed, PTEN associates with E-cadherin-based junctional complexes in several epithelial cell types (Kotelevets *et al*., 2005; Vogelmann *et al*., 2005), which indicates that PI3-kinase signalling at cell–cell contacts is normally subject to tight spatiotemporal regulation. Since Gab2 localizes to cell–cell junctions in MCF-10A acini, and co-immunoprecipitates with E-cadherin, we hypothesize that it may oppose PTEN by acting as a positive regulator of PI3-kinase at cell–cell contact sites. While this does not alter E-cadherin-based adhesion when Gab2 is expressed

alone, in cells exhibiting chronic PI3-kinase activation due to the presence of v-Src, localized amplification of this pathway by Gab2 causes further destabilization of adherens junctions and acinar disruption.

The finding that p85 binding to Gab2 is required for potentiation of v-Src-driven phenotypic alterations is in contrast to those obtained upon co-expression of Gab2 with activated erbB2 in MCF-10A cells, where the formation of multiacinar, invasive structures was dependent on Shp2, but not p85, binding by Gab2 (Bentires-Alj *et al*., 2006). Therefore, distinct effector pathways can be utilized by Gab2 to promote aberrant phenotypes in three-dimensional culture, depending on the co-expressed tyrosine kinase. However, the ability of Gab2 to increase the migratory and invasive potential of v-Src-expressing cells in transwell assays was p85 independent. This effect is likely to be mediated via Shp2, since Shp2-binding defective mutants of Gab1 and Gab2 confer impaired migratory responses to specific stimuli (Yu *et al*., 2002; Ren *et al*., 2004) and Shp2 overexpression increases the migration and invasion of MCF-7 breast cancer cells (Wang *et al*., 2005). Therefore, in the presence of activated Src, Gab2 enhances dissolution of cell–cell contacts and migration/invasion via different binding partners.

Our findings, and those of others, that Gab2 can promote cellular migration and invasion *in vitro* and metastasis in transgenic models of mammary tumourigenesis (Bentires-Alj *et al*., 2006; Ke *et al*., 2007) is of interest in light of the presence of Gab2 in a gene expression signature associated with lymph node metastasis in breast cancer patients (Huang *et al*., 2003). Consequently, it will be important to determine Gab2 expression at different stages during the development of human breast cancer, and to evaluate whether Gab2, either alone or in combination with specific tyrosine kinases, represents a prognostic marker in this disease.

Materials and methods

Plasmids and retroviral vectors
The expression vectors pMIG/Gab2 and its derivatives pMIG/Gab2Δp85 and pMIG/Gab2ΔShp2 were previously described (Brummer *et al*., 2006). The vectors pBABEpuro/c-Src, pBABEpuro/c-SrcY527F and pBABEhygro/v-Src were kind gifts from Wally Langdon (University of Western Australia). The v-Src cDNA was excised from pBABEhygro/v-Src by *BamH*I/*Eco*RI digestion and subsequently subcloned into *BamH*I/*Eco*RI linearized pBABEpuro to yield pBABEpuro/v-Src. These constructs were confirmed by DNA sequencing.

Cell culture and retroviral infection of MCF-10A cells
MCF-10A cells expressing the ecotropic retroviral receptor were maintained and infected with retroviruses as previously described (Debnath *et al*., 2003a; Brummer *et al*., 2006). GFP-positive cells were sorted by flow cytometry, expanded and infected with pBABEpuro/vector, /c-Src, /v-Src or /c-SrcY527F retroviruses produced as described previously (Brummer *et al*., 2006). Cells were selected using puromycin ($2\,\mu g\,ml^{-1}$; Sigma, Castle Hill, NSW, Australia) over 10–14 d. Three-dimensional cultures were established as previously described (Debnath *et al*., 2003a; Brummer *et al*., 2006).

Antibodies
The commercially available antibodies used in this study were described previously (Brummer *et al*., 2006) and in addition include anti-HA polyclonal antibody (Covance, Richmond, CA, USA), anti-v-Src (Ab-1) (Merck Pty Ltd., Kilsyth, Vic, Australia), anti-α-catenin (BD Transduction Laboratories, North Ryde, NSW, Australia) and anti-Laminin V (Millipore, North Ryde, NSW, Australia).

Three-dimensional culture analysis and confocal microscopy
Acini were photographed using a Leica DFC280 camera with Leica Firecam software (Version 1.9). Acinar diameter was measured using ImageJ software (Version 1.34s). Analysis by confocal microscopy was performed as previously described (Debnath *et al*., 2003a; Brummer *et al*., 2006).

Cell lysis of monolayer and three-dimensional Matrigel cultures
Day 4 Matrigel cultures were recovered as previously described (Brummer *et al*., 2006) and lysed with RIPA buffer (50 mM HEPES (pH 7.4), 1% (v/v) Triton-X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM sodium fluoride, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, $10\,\mu g\,ml^{-1}$ aprotinin, $10\,\mu g\,ml^{-1}$ leupeptin and 1 mM sodium orthovanadate). Cells grown in monolayer culture were lysed with RIPA buffer or Triton lysis buffer (0.5% Triton X-100, 25 mM Tris pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, 50 mM sodium fluoride, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, $10\,\mu g\,ml^{-1}$ aprotinin, $10\,\mu g\,ml^{-1}$ leupeptin and 1 mM sodium orthovanadate). Triton-insoluble pellets were resuspended in $2\times$ SDS sample buffer. Characterization of Gab2 binding mutants and immunoprecipitations were performed as previously described (Brummer *et al*., 2006).

Trypsin sensitivity assay
To assay levels of surface E-cadherin, confluent cells were incubated at 37 °C with 0.01% crystalline trypsin (Sigma) in Hanks' balanced salt solution (Sigma) containing either 2 mM $CaCl_2$ or 2 mM EDTA for 30 min. Trypsinized cells were pelleted and lysed in $3\times$ SDS sample buffer. Total cellular protein levels were determined in a corresponding untreated control, lysed in RIPA buffer.

Western blot analysis
This was performed as described previously (Janes *et al*., 1994).

Adhesion assay
Homophilic adhesion to substrata coated with the extracellular domain of human E-cadherin fused to the Fc portion of human IgG (hE/Fc) was measured by resistance to detachment as previously described (Verma *et al*., 2004; Shewan *et al*., 2005).

In vitro *migration/invasion assays*
In vitro migration/invasion assays were performed using cell culture inserts with PET filters, 8 μm pore size, for 24-well plates (BD Falcon, North Ryde, NSW, Australia). The underside of the filter was coated with $0.5\,mg\,ml^{-1}$ Collagen type 1 (Cohesion, Palo Alto, CA, USA). For invasion assays, the upper side of the filter was also coated with Matrigel (1:10 dilution in DMEM F12). The lower compartment of each well contained DMEM F12 with 2% horse serum and the upper chamber contained resuspended cell solution at 2×10^5 cells

per ml in DMEM F12. Cells were incubated for 18 h, after which the remaining cells in the upper chamber were removed. The filter was stained using Diff-Quick System (Lab Aids Pty Ltd, Narabeen, NSW, Australia). Six random fields of migrated/invaded cells were counted under a $\times 10$ objective and each assay was performed in duplicate.

Densitometry and statistical analysis

Densitometry was performed using IP Lab Gel software (Signal Analytics Inc., Vienna, VA, USA). For statistical analyses, we used two-sided Student's *t*-test. Differences were considered to be statistically significant at $P<0.05$. For adhesion assay, data were analysed using a one-way ANOVA with a Newman–Keuls multiple comparison test.

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